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(54) Title: EUCARYOTIC EXPRESSION OF STEROID RECEPTOR PROTEINS

(57) Abstract

Eucaryotic steroid receptor proteins, including human estrogen receptor proteins, are prepared by the expression of a recombinant DNA molecule introduced into appropriate eucaryotic host cells.

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EUCARYOTIC EXPRESSION OF STEROID RECEPTOR PROTEINS

Technical Field

The invention relates to production of desired proteins using recombinant technology. Specifically, it concerns production of steroid receptor proteins employing eucaryotic hosts and expression systems compatible with these hosts.

Background Art

Steroid hormone receptors are well known to regulate gene expression in eucaryotic cells. The action of steroid hormones in vertebrates involves interaction of the specific intracellular steroid receptor protein (SR) with the genome, and in addition, steroid hormones and SR proteins are involved in the regulation of tumor growth in many instances (Lippmann, M.E., Breast Cancer: Trends in Research and Treatment (Raven, NY 1975); Lippmann, M.E., et al, Nature (1975) 256:592). It is believed that binding of the steroid converts the SR protein to a form which binds strongly to nuclear DNA, although the nature of this interaction and the location of the binding is not understood.

It is known that steroid receptor protein, specifically estrogen receptor (ER) is distributed in a tissue-specific manner and the expression of this protein is developmentally regulated. ER from several sources has been purified and shown to have a molecular weight of 65-70 kd (Redeuilh, C., et al Eur J Biochem (1980) 106:481; Lubahn, D.B., et al, J Biol Chem (1985) 260:2215; Katzenellenbogen, B.S., et al, J Biol Chem (1983) 258:3487; Sakai, D., et al, Endocrinol (1984) 115:2379; van Oosberee, T.R., et al, Anal Biochem (1984)

136:321). In addition, related human glutocortocoid receptor (GR) cDNA has been prepared (Hollenberg, S.M., et al, Nature (1985) 318:635).

Recently, the cDNA encoding human estrogen receptor (hER) was isolated from randomly primed λgt10 and λgt11 cDNA libraries prepared from the human breast cancer cell line, MCF-7, and screened with monoclonal anti-ER antibodies and with synthetic oligonucleotides corresponding to two peptide sequences obtained from MCF-7 human ER. This work is reported in Walter, P., et al, Proc Natl Acad Sci (USA) (1985) 82:7889-7893, which is incorporated herein by reference. Among the cDNA clones isolated by oligonucleotide hybridization was a 2.1 kb cDNA clone designated OR8 which cross-hybridized with all other cDNAs and which contained the expected sequences for the two ER peptides. In addition, this cDNA hybridized selectively to a 6.2 kb poly-A RNA which, when translated *in vitro* in the presence of ^{35}S-methionine, was shown to encode the synthesis of immunoreactive 65 kd hER as well as a smaller amount of immunoreactive 46 kd protein. The OR8 cDNA insert was long enough to contain the entire coding sequence for the 65 kd protein.

In the invention herein, the OR8 cDNA clone was successfully incorporated into an expression system compatible with mammalian host cells, and recombinant hER produced using transformed mammalian cell cultures. The availability of expression systems for hER and other steroid receptor proteins provides the ability to produce large amounts of purified steroid receptor proteins, and their production in eucaryotic hosts assures that post-translational processing and folding which is capable of regenerating material closely

analogous to the natively produced intracellular protein will be obtained.

Disclosure of the Invention

The invention provides the complete DNA sequence encoding human estrogen receptor protein (hER) along with the deduced amino acid sequence. The availability of this sequence information permits comparisons with related receptor proteins such as the glucocortocoid receptor and the v-erb-A (AEV) oncogene product.

In addition, the pertinent coding sequence has been incorporated into an expression system operable in eucaryotic host cells, and specifically mammalian host cells. This permits expression of the sequences under conditions which favor appropriate post-translational processing, and provides quantities of protein useful for design of agonist and antagonist compounds, for study of the mechanism of action of the steroid binding proteins in general, and for use in diagnostic assays for SR proteins themselves or for antibodies raised against them.

Thus, in one aspect, the invention relates to an expression system for a vertebrate steroid receptor protein which contains DNA encoding the receptor protein operably linked to control sequences capable of expressing these sequences in eucaryotic host cells. In additional aspects, the invention relates to recombinant host eucaryotic cells containing the expression system of the invention, to methods of producing steroid receptor proteins using these cells, and to these steroid receptor proteins so produced.

Brief Description of the Drawings

Figure 1 shows the cDNA sequence encoding human estrogen receptor protein and the deduced amino acid sequence.

Figure 2 shows comparative amino acid sequences for hER, human GR, and a putative AEV oncogene protein.

Figure 3 shows sedimentation analysis of human estrogen receptor expressed by OR8 cDNA in CHO K-1 cells.

Modes of Carrying Out the Invention

A. Definitions

"Expression system" refers to a collection of components as subsequently designated and may include, as specified, only a coding sequence operably linked to control sequences, to these sequences further linked to an enhancer, to a vector containing these, and any further DNA sequence relevant to affecting expression.

"Human metallothionein-II" promoter (hMT-II) refers to control sequences derived from the human MT-II gene or their functional equivalents. The control sequences of this gene are described in detail by Karin, M., et al, Nature (1982) 299:797-802.

"Chinese Hamster Ovary" (CHO) cells include the standard cell line ATCC CCL-61, and its relatives isolated from the same source tissue, as well as derivatives thereof. Derivatives are mutants of the line which may differ genotypically or phenotypically from the original line, but which are obtained therefrom by intentional or inadvertent mutation.

"Derived from" as it pertains to, for example, DNA or protein sequences refers to similarity in structure and not necessarily to physical derivation.

"Purified" or "pure" refers to material which is free from substances which normally accompany it as found in its native state. Thus "pure" SR, for example, refers to SR which does not contain materials normally associated with its in situ environment in human cells. Of course, "pure" SR may include materials in specific association with it, such as its glycoside residues.

"Operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, control sequences or promoters operably linked to a coding sequence are capable of effecting the expression of the coding sequence.

"Control sequence" refers to a DNA sequence or sequences which are capable, when properly ligated to a desired coding sequence, of effecting its expression in hosts compatible with such sequences. Such control sequences include promoters and termination signals. Additional factors necessary or helpful in effecting expression such as enhancers may also be identified. As used herein, "control sequences" simply refers to whatever DNA sequence may be required to effect expression in the particular host used.

"Cells" or "cell cultures" or "recombinant host cells" or "host cells" are often used interchangeably as will be clear from the context. These terms include the immediate subject cell, and, of course, the progeny thereof. It is understood that not all progeny are exactly identical to the parental cell, due to chance mutations or differences in environment. However, such altered progeny are included in these terms, so long as the progeny retain the characteristics relevant to those conferred on the originally transformed cell.

8. General Description

The invention herein represents the first expression of a steroid receptor protein under environmental conditions which are conducive to mimicing the native product. To effect such expression, the cDNA, genomic DNA or other DNA encoding the desired vertebrate steroid receptor protein is placed under control of control sequences operable in eucaryotic cells for expression. The invention is illustrated below with respect to human estrogen receptor; however, it is recognized that using expression systems of the invention, including that specifically illustrated, other steroid receptors may be successfully produced.

In order to obtain a valid construction of the native protein, it is desirable that the production be effected in eucaryotic hosts. Common eucaryotic hosts include yeast and mammalian cells. A particular expression system for mammalian cells is illustrated below; however, alternate expression systems are also available and could be employed with suitable modifications to obtain expression of appropriate steroid receptor encoding sequences.

Eucaryotic microbes, though less desirable than mammalian cultures may be used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most used although a number of other yeast strains are commonly available. Vectors employing, for example, the 2 μ origin of replication of Broach, J. R., Meth Enz (1983) 101:307, or other yeast compatible origins of replications (see, for example, Stinchcomb, et al, Nature (1979) 282:39, Tschempe, et al, Gene (1980) 10:157 and Clarke, L, et al, Meth Enz (1983) 101:300) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic

enzymes (Hess, et al, J Adv Enzyme Reg (1968) 7:149; Holland, et al, Biochemistry (1978) 17:4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman, et al, J Biol Chem (1980) 255:2073), and those for other glycolytic enzymes. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

Transformations into yeast may be carried out according to the method of Van Solingen, P., et al, J Bact (1977) 130:946 or of Hsiao, C. L., et al, Proc Natl Acad Sci (USA) (1979) 76:3829.

Mammalian host cell expression is, however preferred, and general techniques for such expression are known. See, for example, Axel, et al, 4,399,216. These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al, Nature (1978) 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papiloma virus, or avian sarcoma viruses. The controllable promoter, hMTII

(Karin, M., et al, Nature (1982) 299:797-802) may also be used. It now appears, also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions; these are also illustrated below. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes. For mammalian cells the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, optionally as modified by Wigler, M., et al, Cell (1979) 16:777-785 may be used.

To effect the desired expression in eucaryotic hosts, the gene sequences are ligated into suitable control sequences transformed into the compatible host cells and the cell cultures grown under appropriate conditions. If the control sequences include inducible promoters, appropriate induction conditions are supplied. As the steroid receptor proteins are intracellular, they lack signal sequences and are normally preceded by a methionine start codon. Alternate constructions which delete this codon and include heterologous signal sequences capable of effecting the secretion of this protein from the host are also capable of construction. The signal sequences from, for example, normally secreted mammalian proteins such as renin, growth hormone, or insulin are known in the art and could be used. If for expression in yeast, yeast signal sequences such as those associated with α factor can be employed.

C. Utility

The availability of significant amounts of purified steroid receptor protein is advantageous in both diagnostic assays and in designing means for regulating steroid mediated metabolism. In connection with the former, it may be desirable to assess the levels of receptor present in various tissues as a diagnostic tool for disorders of steroid regulated metabolism, and also to assess the susceptibility of various tumors to control by regulation of steroid supply. In addition, it is possible to raise antibodies specific for steroid receptors, and the availability of large amounts of antigen facilitates the assays for them.

Thus, the desirability of obtaining pure recombinant human estrogen receptor and other steroid hormone receptors has several facets. First, milligram amounts of the material are obtainable using this procedure. Milligram amounts are capable of crystallization to permit three dimensional studies using X-ray diffraction and computer analysis. This permits deductions concerning the shape of the molecule, thus defining proper shapes for substances useable as agonists and antagonists of the hormone. Agonists and antagonists are critically important in regulating those aspects of metabolism mediated by steroids, including reproductive function, inflammatory responses, blood pressure, and secondary sex characteristics. The responsiveness of some tumors to steroids is also known, and is reinforced by the disclosure herein of regional homology between human ER, glucocortocoid receptor, and a putative v-erb-A oncogene product.

ER and other steroid receptors have DNA and steroid binding domains, and agonists or antagonists to steroid activity are substances whose interactions with

SR are stabilized by design of the 3-dimensional structures so as to enhance the ability of the substance to interact positively and specifically with a particular region of the SR. This lock and key spatial arrangement will result from molecules designed complementary to the surface contours of the crystallized ER or other steroid receptor of the invention. It is understood that "surface" includes convolutions which may face inward, and specifically includes both normal sites of interaction and internal structure which results in these sites. Furthermore, "complementary" is understood to mean that in addition to spatial conformations which "fit", interactions between the receptor and the molecule which matches its surface contours are attractive and positive. These interactions may be hydrogen bonding, ionic, or hydrophobic affinity. Antagonists and agonists are modified steroids complementary to the 3-D structure.

Second, even without the assistance of a three dimensional structure determination, purified receptors of the invention are of significance as reagents in screening agonists and antagonists <u>in vitro</u> as an ad hoc approach to evaluation. Impure receptor preparations currently available yield confusing data due to the impact of the impurities on the test results. For example, contaminants which turn out to be themselves agonists or antagonists for steroids will interfere with the evaluation. Thus, a substantial improvement in current screening techniques for agonists and antagonists would be effected by the availability of the purified human receptor protein.

In addition, because the availability of materials for recombinant production of a protein permits sequence modification, for example, by

site-specific mutagenesis, modification of characteristics of the product receptor, such as its ligand binding specificity is possible. Therefore the recombinant vectors of the invention provide the starting materials to obtain a series of modified receptors for a spectrum of processing conversions.

Finally, the recombinant receptor of the invention is also useful in providing a specific and sensitive diagnostic assay for human receptor protein in biological samples. Such assays are important in, for example, diagnosis of tumor sensitivity to steroid metabolism. The availability of purified recombinant human receptor will provide this material for standardization and calibration of direct immunoassay.

D. Examples

Illustrated below is the construction of an expression system effective in producing recombinant human estrogen receptor. The examples are not to be construed as limiting the invention.

Example I

Characterization of cDNA Encoding Human Estrogen Receptor

OR8 cDNA, obtained as described by Walter, P., et al, (supra), was subcloned into the EcoRI site of M13mp9 and clones containing both orientations of cDNA were isolated and sequenced by the method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463; Schreirer, P.H., et al, J Mol Biol (1979) 129:169. The sequence obtained is shown in Figure 1. The open reading frame containing 1785 nucleotides corresponds to 595 amino acids and a calculated Mr of 66,200. Peptide sequences from purified hER are found throughout the

open reading frame and the location of the ATG start codon was surmised because it is the first to appear downstream from the in frame terminator TGA at nucleotides -54 to -52. Comparison of the deduced amino acid sequence with the known sequences of human glucocortocoid receptor (GR) and to the product of the v-erb-A (AEV) oncogene showed strong homology in a region rich in cysteine, lysine and arginine, about 300-350 amino acids from the carboxy terminus of each. Extensive homologies are found in various corresponding regions of these proteins as shown in Figure 2.

Example 2

Construction of an Expression System

The host expression vector contains the metallothionein-II promoter system (hMT-II) which is inducible in the presence of zinc ion, a viral enhancer, and about 600 bp of the 3' untranslated region of human growth hormone. The expression vector is obtained by replacing the human growth hormone encoding sequence from the vector designated phGHg-SV(10) which is constructed as described below. An alternate vector, also described, phGHg-SV(9) could also be used.

The construction of these vectors which are used as sources for the host vector sequences is as follows:

The Promoter Sequences

The plasmid pHS1 contains 840 bp of the hMT-II sequence from p84H (Karin, M., et al, Nature (1982) 299:297-302) which spans from the HindIII site at position -765 of the hMT-II gene to the BamHI cleavage site at base + 70. Plasmid p84H was digested to completion with BamHI, treated with exonuclease Bal-31

to remove terminal nucleotides, and then digested with HindIII. The desired 840 bp HindIII/blunt fragment was ligated into pUC8 (Vieira, J., et al, Gene (1982) 19:259-268) which had been opened with HindIII and HincII digestion. The ligation mixture was transformed into E. coli HB101 to Amp^R, and one candidate plasmid, designated pHS1, was isolated and sequenced by dideoxy sequencing. pHS1 contains the hMT-II control sequences upstream of a polylinker containing convenient restriction sites.

The hGH Sequence

Genomic sequences encoding hGH were isolated from p2.6-3 (DeNoto, et al, Nucleic Acids Res (1981) 19:3719)by digestion with BamHI, which cuts at the 5' end of the first exon, and EcoRI, which cuts 3' of the functional gene, followed by polyacrylamide gel purification. The isolated fragment was ligated into BamHI/EcoRI digested pHS1 and the ligation mixture transformed into E. coli MC1061 to Amp^R. Successful transformants were screened by restriction analysis, and a strain containing the desired plasmid, designated pMT-hGHg was further propagated to prepare quantities of plasmid DNA.

The Enhancer Sequence

A pair of host expression vectors containing the SV40 enhancer in operable linkage to the MT-II promoter and the 3' untranslated sequences from hGH was constructed by inserting an 1120 bp SV40 DNA fragment into the HindIII site preceding the MT-II promoter sequences in pMT-bGHg. The SV40 DNA fragment spans the SV40 origin of replication and includes nucleotide 5171 through nucleotide 5243 (at the origin), the duplicated

72 bp repeat from nucleotide 107-250, and continues through nucleotide 1046 on the side of the origin containing the 5' end of late viral mRNAs. This HindIII 1120 bp fragment is obtained from a HindIII digest of SV40 DNA (Buchman, A.R., et al, DNA Tumor Viruses, 2d ed (J. Tooze, ed.), Cold Spring Harbor Laboratory, New York (1981), pp. 799-841), and cloned into pBR322 for amplification. The cloning vector was cut with HindIII, and the 1100 bp SV40 DNA fragment isolated by gel electrophoresis and ligated into HindIII-digested, CIP-treated, pMT-hGHg. The resulting vectors, designated phGHg-SV(9) and phGHg-SV(10), contain the fragment in opposite orientations preceding the MT-II promoter. In phGHg-SV(9), the enhancer is about 1600 bp from the 5' mRNA start site; in the opposite orientation it is approximately 980 bp from the 5' mRNA start site. Both orientations are operable, but the orientation wherein the enhancer sequences are proximal to the start site provides higher levels of expression. It is believed that deletions which place the enhancer 250-400 bp upstream of the transcription start are optimal.

Insertion of hER cDNA

phGHg-SV(10), prepared as above, was digested with BamHI and SmaI to delete the hGH encoding sequences. (BamHI cleaves in the polylinker sequence; SmaI in the 3' untranslated region of hGH, leaving about 600 bp in the vector fragment.)

The vector fragment was blunted using Klenow and the 4 dNTPs and ligated with the blunted EcoRI 2.1 kb OR8 fragment described above containing the estrogen receptor. The resulting vector, designated phER-SV(10) was cloned in E. coli, and then transformed into CHO-K1 cells and successful transformants isolated.

Example 3

Production of Recombinant hER

Successful transformant colonies were grown in monolayer culture to confluency and a zinc-supplemented DMEM/Ham's F-12 medium (Ham, R.G., Proc Natl Acad Sci (USA) (1965) 53:288), released with EDTA, and homogenized by Polytron disruption in a buffer containing 10 mM Tris (pH 7.4) and 20 mM sodium molybdate. The homogenates were centrifuged at 250,000 x g for 30 min and the supernatant fractions were then labeled with 0.5 nM estradiol (57 Ci/mmol) with or without a 200 fold excess of non-radioactive control estradiol or diethylstilberterol for 60 min at 4°C.

In a separate experiment, aliquots (200 µl) of labeled extract were incubated for 60 min at 4°C either in the presence or absence of rat monoclonal ER antibody (10 µg of $D75P3_{\gamma}$, $D547Sp_{\gamma}$ or $H222Sp_{\gamma}$ in a final volume of 220 µl).

For sedimentation analyses. 200 µl aliquots of labeled extract or incubate were layered onto linear 10-30% sucrose gradients (3.5 ml) prepared in 10 mM Tris, 10 mM sodium molybdate, 1.5 mM EDTA, pH 7.4 and either 10 mM KCl (low salt) or 400 mM KCl (high salt) and centrifuged at 0°C for 15 hr at 253,000 x g. Successive 100 µl fractions were collected and radioactivity was measured in Triton X-100 toluene scintillation mixture at 35% counting efficiency. C^{14}-labeled ovalbumin (3.6 S) and C^{14}-labeled IgG (7.0 S) were used as sedimentation markers in parallel gradients.

Figure 3 shows the results of the sedimentation analyses. Panel A shows the profiles in low salt gradients of the extracts from hER transformed cells (triangles) or extracts from untransformed cells

(circles) labeled with 0.5 nM estradiol. These results indicate the presence of a labeled estradiol receptor complex which sediments at 8-9 S.

Panel B shows the corresponding profiles as obtained in high salt gradients. The estradiol labeled receptor protein from the transformed cells as shown (triangles) now has a sedimentation value of 4 S. This verifies the production of ER as the formation of an 8-10 S salt sensitive hormone receptor complex in hypotonic extracts of responsive cells is considered to be a diagnostic characteristic of steroid receptors.

Further verification is also shown in Figure 3. When the homogenates were labeled with 0.5 nM labeled estradiol/100 nM cold estradiol (closed circles) the estrogen receptor complex was abolished. Figure 3 also shows that the complex incubated with the anti-receptor antibody D75P_γ (open circles) shifts the sedimentation to an 8 S complex. In addition, the concentration of the estrogen receptor complex was more than doubled by the inclusion of 10^{-4} M zinc ion in the culture medium 24 hr prior to cell harvest, consistent with the induction of the metallothionein promoter by zinc (data not shown). The foregoing sedimentation characteristics are entirely consistent with the expression of hER by the transformed cells.

Claims

1. An expression system for vertebrate steroid receptor protein which comprises a DNA sequence encoding SR operably linked to control sequences compatible with eucaryotic host cells.

2. The expression syscem of claim 1 wherein the host cells are mammalian.

3. The expression system of claim 2 wherein the host cells are CHO cells.

4. The expression system of claim 1 wherein SR is estrogen receptor (ER).

5. The expression system of claim 4 wherein ER is human estrogen receptor (hER).

6. Recombinant host eucaryotic cells which contain the expression system of claim 1.

7. The host cells of claim 6 which are mammalian.

8. A method to produce recombinant vertebrate SR which comprises culturing the cells of claim 6 and recovering the SR protein.

9. The method of claim 8 wherein the SR is recovered by disrupting the cells, removing cell debris, and recovering clarified lysate.

10. Recombinant vertebrate steroid receptor produced by the method of claim 8.

11. The SR of claim 10 which is estrogen receptor.

12. The ER of claim 11 which is human ER.

FIG. 1-1

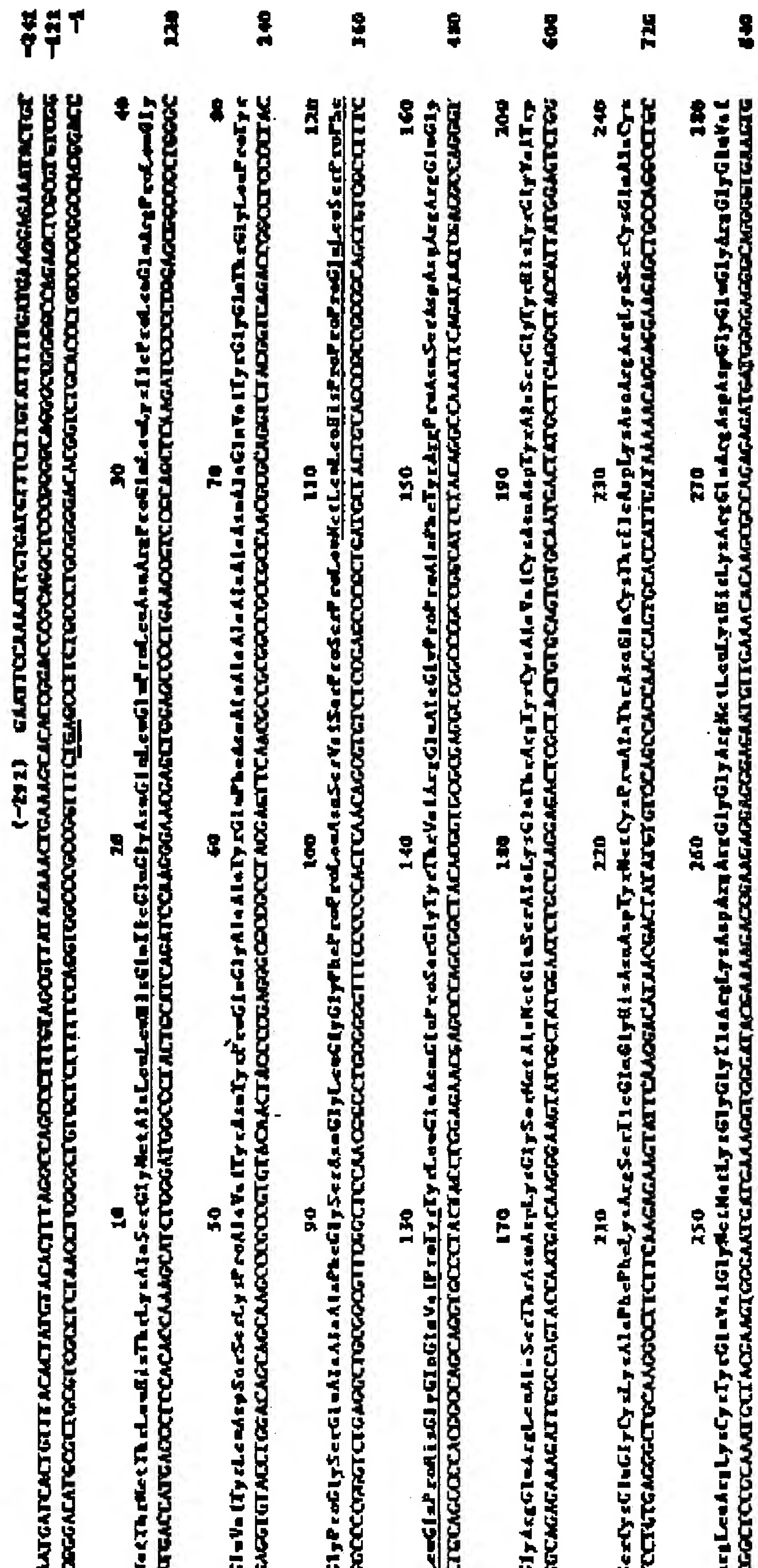

FIG. 1-2

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                       290                            300                            310                            320
GlySerAlaGlyAspMetArgAlaAlaAsnLeuTrpProSerProLeuMetIleLysArgSerLysLysAsnSerLeuAlaLeuSerLeuThrAlaAspGlnMetValSerAlaLeuLeu
GGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCCTTGTTG   960

                       330                            340                            350                            360
AspAlaGluProProIleLeuTyrSerGluTyrAspProThrArgProPheSerGluAlaSerMetMetGlyLeuLeuThrAsnLeuAlaAspArgGluLeuValHisMetIleAsnTrp
GATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACCCTTCAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATGATCAACTGG   1080

                       370                            380                            390                            400
AlaLysArgValProGlyPheValAspLeuThrLeuHisAspGlnValHisLeuLeuGluCysAlaTrpLeuGluIleLeuMetIleGlyLeuValTrpArgSerMetGluHisProVal
GCGAAGAGGGTGCCAGGCTTTGTGGATTTGACCCTCCATGATCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATGATTGGTCTCGTCTGGCGCTCCATGGAGCACCCAGTG   1200

                       410                            420                            430                            440
LysLeuLeuPheAlaProAsnLeuLeuLeuAspArgAsnGlnGlyLysCysValGluGlyMetValGluIlePheAspMetLeuLeuAlaThrSerSerArgPheArgMetMetAsnLeu
AAGCTACTGTTTGCTCCTAACTTGCTCTTGGACAGGAACCAGGGAAAATGTGTAGAGGGCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTC   1320

                       450                            460                            470                            480
GlnGlyGluGluPheValCysLeuLysSerIleIleLeuLeuAsnSerGlyValTyrThrPheLeuSerSerThrLeuLysSerLeuGluGluLysAspHisIleHisArgValLeuAsp
CAGGGAGAGGAGTTTGTGTGCCTCAAATCTATTATTTTGCTTAATTCTGGAGTGTACACATTTCTGTCCAGCACCCTGAAGTCTCTGGAAGAGAAGGACCATATCCACCGAGTCCTGGAC   1440

                       490                            500                            510                            520
LysIleThrAspThrLeuIleHisLeuMetAlaLysAlaGlyLeuThrLeuGlnGlnGlnHisGlnArgLeuAlaGlnLeuLeuLeuIleLeuSerHisIleArgHisMetSerAsnLys
AAGATCACAGACACTTTGATCCACCTGATGGCCAAGGCAGGCCTGACCCTGCAGCAGCAGCACCAGCGGCTGGCCCAGCTCCTCCTCATCCTCTCCCACATCAGGCACATGAGTAACAAA   1560

                       530                            540                            550                            560
GlyMetGluHisLeuTyrSerMetLysCysLysAsnValValProLeuTyrAspLeuLeuLeuGluMetLeuAspAlaHisArgLeuHisAlaProThrSerArgGlyGlyAlaSerVal
GGCATGGAGCATCTGTACAGCATGAAGTGCAAGAACGTGGTGCCCCTCTATGACCTGCTGCTGGAGATGCTGGACGCCCACCGCCTACATGCGCCCACTAGCCGTGGAGGGGCATCCGTG   1680

                       570                            580                            590
GluGluThrAspGlnSerHisLeuAlaThrAlaGlySerThrSerSerHisSerLeuGlnLysTyrTyrIleThrGlyGluAlaGluGlyPheProAlaThrValSTOP
GAGGAGACGGACCAAAGCCACTTGGCCACTGCGGGCTCTACTTCATCGCATTCCTTGCAAAAGTATTACATCACGGGGGAGGCAGAGGGTTTCCCTGCCACAGTCTGAGAGCTCCCTGGC   1800
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FIG. 2

h-GR	405	pro	pro	ser	ser	ser	ser	thr	ala	thr	thr	gly	pro	pro	pro	lys	leu
h-ER	169	asn	asp	lys	gly	ser	met	ala	met	gln	ser	ala	lys	gln	thr	arg	tyr
v-erb-A	21	ser	ser	met	ser	gly	tyr	ile	pro	ser	cys	leu	asp	lys	asp	glu	gln
h-GR	421	cys	leu	val	cys	ser	asp	glu	ala	ser	gly	cys	his	tyr	gly	val	leu
h-ER	185	cys	ala	val	cys	asn	asp	tyr	ala	ser	gly	tyr	his	tyr	gly	val	trp
v-erb-A	37	cys	val	val	cys	gly	asp	lys	ala	thr	gly	thr	his	tyr	arg	cys	ile
h-GR	437	thr	cys	gly	ser	cys	lys	val	phe	phe	lys	arg	ala	val	gln	gly	—
h-ER	201	ser	cys	glu	gly	cys	lys	ala	phe	phe	lys	arg	ser	ile	gln	gly	—
v-erb-A	53	thr	cys	glu	gly	cys	lys	ser	phe	phe	arg	arg	thr	ile	gln	lys	asn
h-GR	452	gln	his	asn	—	tyr	leu	cys	ala	gly	arg	asn	asp	cys	ile	ile	asp
h-ER	216	—	his	asn	asp	tyr	met	cys	pro	ala	thr	asn	gly	cys	thr	ile	asp
v-erb-A	69	leu	his	pro	thr	tyr	ser	cys	thr	tyr	asp	gly	cys	cys	val	ile	asp
h-GR	467	lys	ile	arg	arg	lys	asn	cys	pro	ala	cys	arg	tyr	arg	lys	cys	leu
h-ER	231	lys	asn	arg	arg	lys	ser	cys	gln	ala	cys	arg	leu	arg	lys	cys	tyr
v-erb-A	85	lys	ile	thr	arg	asn	gln	cys	gln	leu	cys	arg	phe	lys	lys	cys	ile
h-GR	483	gln	ala	gly	met	asn	leu	glu	ala	arg	lys	thr	lys	lys	lys	ile	lys
h-ER	247	glu	val	gly	met	met	lys	gly	gly	ile	arg	lys	asp	arg	arg	gly	gly
v-erb-A	101	ser	val	gly	met	ala	met	asp	leu	val	leu	asp	asp	ser	lys	arg	val

FIG. 3B

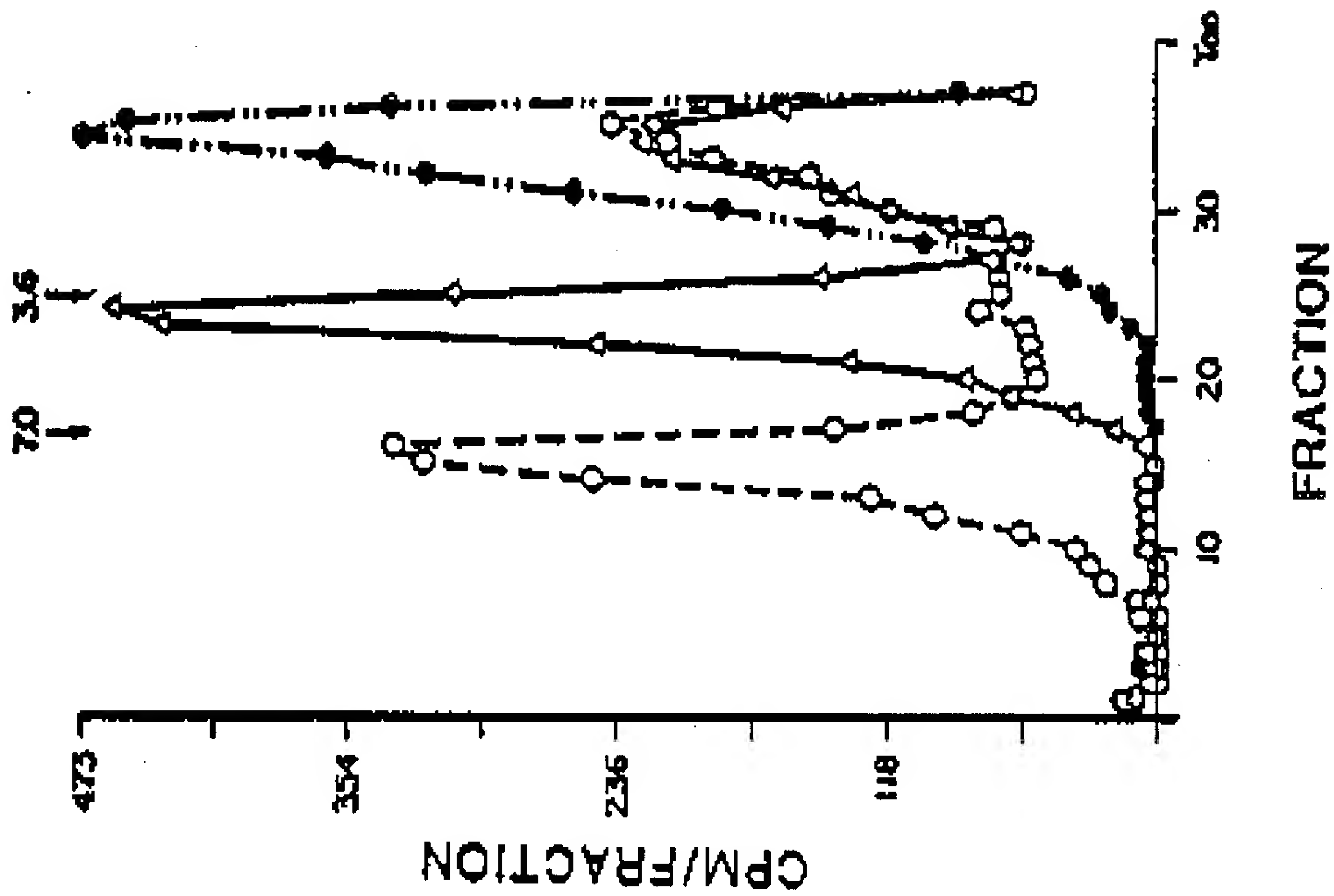
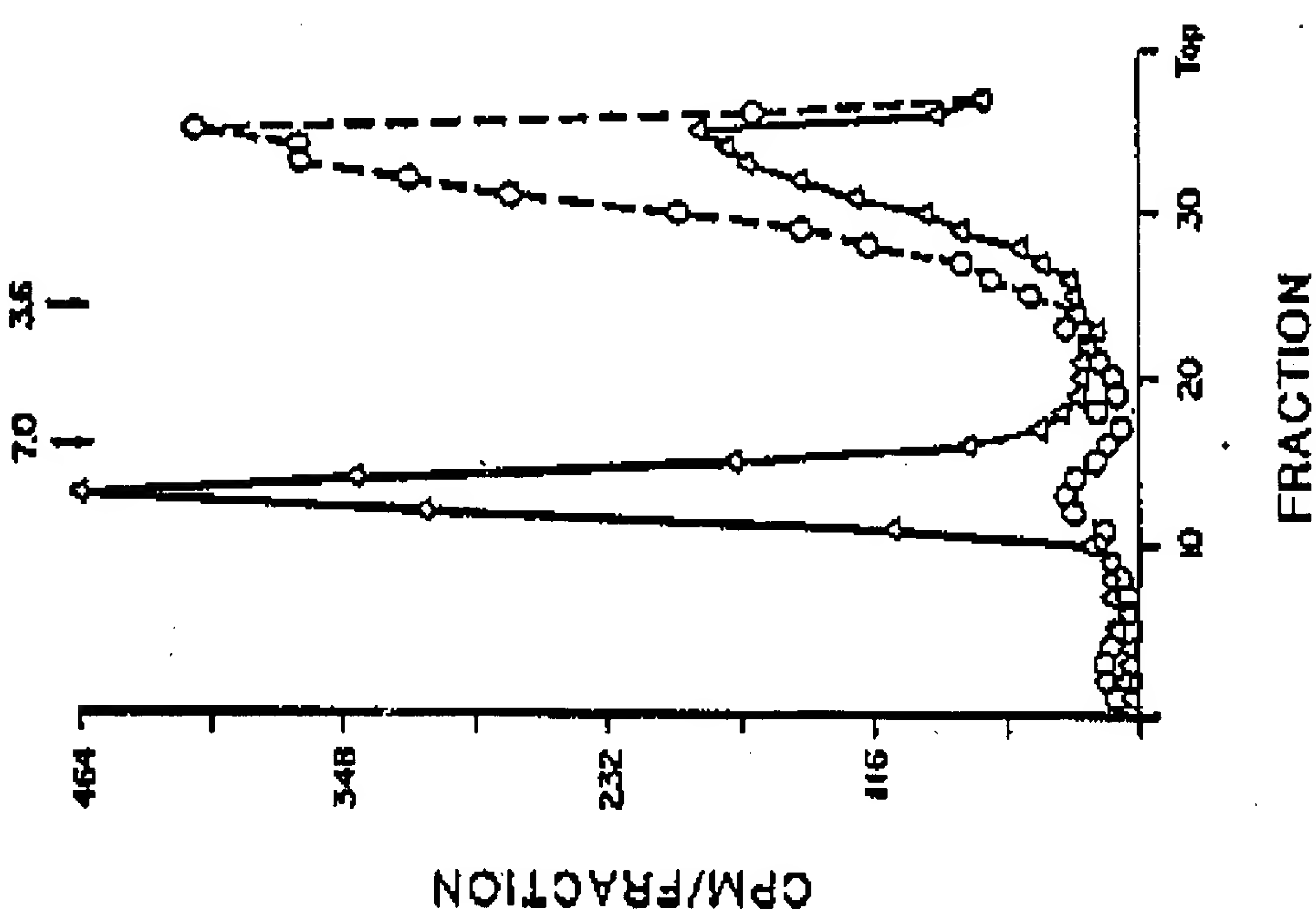

7.0
3.6
473
354
236
118
CPM/FRACTION
10
20
30
Top
FRACTION

FIG. 3A


7.0
3.6
464
348
232
116
CPM/FRACTION
10
20
30
Top
FRACTION

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00341

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 21/00, 21/02; C12N 5/02; C07K 13/00

U.S. 435/68, 70; 435/240; 530/350

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68, 70, 172.3, 240 935/11, 13 530/350

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS DATA BASE (CAS) 1967-1987, BIOLOGICAL ABSTRACTS DATA BASE (BIOSIS) 1967-1987. Keywords: steroid, receptor, recombinant, plasmid.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,P	CHEMICAL ABSTRACTS (COLUMBUS, OHIO, USA) Volume 105, No. 7, issued 18 August 1986 (CHAMBON ET AL) "Steroid hormone receptor genes: cloning, organization and expression", see page 156, column 1, the abstract No. 55502g, ICSU SHORT REPORT, 1986, 4, 246-249 (Eng).	1-12
X,P	CHEMICAL ABSTRACTS (COLUMBUS, OHIO, USA) Volume 105, No. 1, issued 07 July 1986 (GREEN ET AL) "Cloning of the human estrogen receptor cDNA", see pages 164-165, columns 2 and 1, respectively, the abstract No. 1670s, JOURNAL STEROID BIOCHEMISTRY, 1986, 24(1), 77-83 (Eng).	1-12
X	PROCEEDINGS NATIONAL ACADEMY SCIENCES (WASHINGTON, D.C.) Volume 82, issued December 1985 (WALTER ET AL) "Cloning of the human estrogen receptor cDNA". See pages 7889-7890.	1-12

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
15 April 1987	24 APR 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Thomas D. Mays

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	NATURE (LONDON) Volume 318, issued 19 December 1985 (HOLLENBERG ET AL) "Primary structure and expression of a functional human glucocorticoid receptor cDNA". See pages 636-638.	1-12
Y	US, A, 4,232,001 (JENSEN ET AL) 04 November 1980. See columns 3-6.	1-12

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	BIOCHEMICAL SOCIETY TRANSACTIONS (LONDON) Volume 12, No. 3, issued 1984 (GILNA ET AL). "A strategy for the cloning of the human estrogen receptor gene: optimization of an immunodetection system for use in the screening of a cDNA library". See page 486.	1-12